Articles

Treatment of Ebola virus infection with a recombinant inhibitor of factor VIIa/tissue factor: a study in rhesus monkeys

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Summary

Background Infection with the Ebola virus induces overexpression of the procoagulant tissue factor in primate monocytes and macrophages, suggesting that inhibition of the tissue-factor pathway could ameliorate the effects of Ebola haemorrhagic fever. Here, we tested the notion that blockade of fVIIa/tissue factor is beneficial after infection with Ebola virus.

Methods We used a rhesus macaque model of Ebola haemorrhagic fever, which produces near 100% mortality. We administered recombinant nematode anticoagulant protein c2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation, to the macaques either 10 min (n=6) or 24 h (n=3) after a high-dose lethal injection of Ebola virus. Three animals served as untreated Ebola virus-positive controls. Historical controls were also used in some analyses.

Findings Both treatment regimens prolonged survival time, with a 33% survival rate in each treatment group. Survivors are still alive and healthy after 9 months. All but one of the 17 controls died. The mean survival for the six rNAPc2-treated macaques that died was 11·7 days compared with 8·3 days for untreated controls (p=0·0184). rNAPc2 attenuated the coagulation response as evidenced by modulation of various important coagulation factors, including plasma D dimers, which were reduced in nearly all treated animals; less prominent fibrin deposits and intravascular thromboemboli were observed in tissues of some animals that succumbed to Ebola virus. Furthermore, rNAPc2 attenuated the proinflammatory response with lower plasma concentrations of interleukin 6 and monocyte chemoattractant protein-1 (MCP-1) noted in the treated than in the untreated macaques.

Interpretation Post-exposure protection with rNAPc2 against Ebola virus in primates provides a new foundation for therapeutic regimens that target the disease process rather than viral replication.

Lancet 2003; **362:** 1953–58

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Introduction

Ebola virus causes severe haemorrhagic fever in primates.[1,2] Acute mortality caused by the Zaire species of Ebola virus has been about 80% in outbreaks in human beings[1] and nearly 100% in monkey models of the genus macaca.[2] There are no effective treatments for Ebola virus haemorrhagic fever. Various therapeutic strategies protect rodents from lethal Ebola haemorrhagic fever; however, these strategies have not proven effective in non-human primates,[3–6] suggesting important pathogenic differences between these models.[2,7]

The disease triggered in primates is thought to involve inappropriate or maladaptive host responses, and includes development of coagulation abnormalities not evident in rodents. Although the coagulopathy seen in Ebola haemorrhagic fever is probably caused by multiple factors, data suggest tissue factor plays an important part in triggering the coagulation abnormalities that characterise infections in primates.[8] The exposure of cells that express tissue factor on their surfaces to flowing blood is sufficient to initiate coagulation.[9] Expression of tissue factor can be induced in the endothelium and in monocytes in vitro by various agonists, even though these cells do not constitutively express tissue factor.[9] Previously, we have shown[8] that Ebola virus induces overexpression of tissue factor in primate monocytes and macrophages, and that overexpression depends on viral replication. Overexpression of tissue factor is one of the leading causes of disseminated intravascular coagulation and thrombosis-related organ failure.[10] Therefore, we reasoned that by blocking the pathway leading from the formation of fVIIa/tissue factor to thrombin, we might alter the disease pathogenesis in Ebola virus infections of non-human primates, with the hope that this approach might be useful in augmenting strategies that have protected rodents from lethal infection.

Recombinant nematode anticoagulant protein c2 (rNAPc2) is an 85-aminoacid protein that directly inhibits the fVIIa/tissue factor complex by a unique mechanism that requires initial binding of rNAPc2 to activated or zymogen factor X.[11] The antithrombotic potential of fVIIa/tissue factor inhibition by rNAPc2 has been shown in phase II trials in orthopaedic surgery[12] and coronary revascularisation.[13] We, therefore, used an established rhesus macaque model of Ebola haemorrhagic fever[2] to test the notion that blockade of fVIIa/tissue factor is beneficial after infection with Ebola virus.

Methods

Animals

We inoculated healthy adult rhesus macaques (*Macaca mulatta*) by intramuscular injection with 0·5 mL of viral stock that contained 1000 plaque forming units (PFU) of Ebola virus (Zaire 95 isolate).[3]

Our research was undertaken in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
13 DEC 2003	**N/A**	**-**

4. TITLE AND SUBTITLE
Treatment of Ebola virus infection with a recombinant inhibitor of factor VIIa/tissue factor: a study in rhesus monkeys, Lancet 362:1953-1958

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Geisbert, TW Hensley, LE Jahrling, PB Larsen, T Geisbert, JB Paragas, J Young, HA Fredeking, TM Rote, WE Vlasuk, GP

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
BACKGROUND: Infection with the Ebola virus induces overexpression of the procoagulant tissue factor in primate monocytes and macrophages, suggesting that inhibition of the tissue-factor pathway could ameliorate the effects of Ebola haemorrhagic fever. Here, we tested the notion that blockade of fVIIa/tissue factor is beneficial after infection with Ebola virus. METHODS: We used a rhesus macaque model of Ebola haemorrhagic fever, which produces near 100% mortality. We administered recombinant nematode anticoagulant protein c2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation, to the macaques either 10 min (n=6) or 24 h (n=3) after a high-dose lethal injection of Ebola virus. Three animals served as untreated Ebola virus-positive controls. Historical controls were also used in some analyses. FINDINGS: Both treatment regimens prolonged survival time, with a 33% survival rate in each treatment group. Survivors are still alive and healthy after 9 months. All but one of the 17 controls died. The mean survival for the six rNAPc2-treated macaques that died was 11.7 days compared with 8.3 days for untreated controls (p=0.0184). rNAPc2 attenuated the coagulation response as evidenced by modulation of various important coagulation factors, including plasma D dimers, which were reduced in nearly all treated animals; less prominent fibrin deposits and intravascular thromboemboli were observed in tissues of some animals that succumbed to Ebola virus. Furthermore, rNAPc2 attenuated the proinflammatory response with lower plasma concentrations of interleukin 6 and monocyte chemoattractant protein-1 (MCP-1) noted in the treated than in the untreated macaques. INTERPRETATION: Postexposure protection with rNAPc2 against Ebola virus in primates provides a new foundation for therapeutic regimens that target the disease process rather than viral replication.

15. SUBJECT TERMS **Filovirus, antibodies, Ebola, procoagulant tissue factor, nematodes, efficacy, laboratory animals, nonhuman primates**					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**6**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

animals, and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where the research was done is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Treatment and investigations

We treated rhesus monkeys with subcutaneous injections of rNAPc2 (30 μg/kg bodyweight, once daily) or sterile saline (untreated controls). Treatment began either 10 min or 24 h after challenge with Ebola virus, and continued through to day 14 post exposure and through to day 8 post exposure (with no treatment on post-exposure day 7 because of issues of drug availability), respectively.

The dose of rNAPc2 used and the regimen followed were different in this study than described in clinical studies.[12,13] In the absence of dose-response data, we used the highest dose of rNAPc2 deemed safe in non-human primates. Importantly, the elimination half-life of rNAPc2 in non-human primates is about 18–20 h shorter than in people; therefore, we administered the drug daily instead of every other day as was done in the clinical studies.[12,13] The maximum plasma concentration of rNAPc2 was estimated to be about 1 μg/mL based on previous studies in non-human primates that used the same dosing regimen.

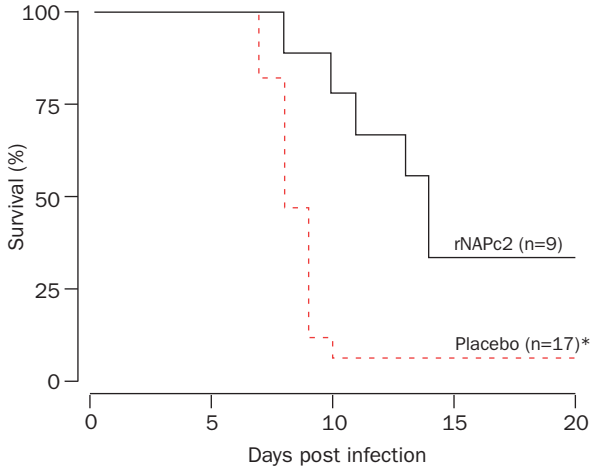
Infectious virus in EDTA plasma was assayed by counting plaques on Vero cells maintained as monolayers in six-well plates under agarose, as previously described.[3]

We measured total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, haematocrit (packed cell volume) values, total haemoglobin, mean cell volume, mean corpuscular volume, and mean corpuscular haemoglobin concentration in blood samples collected in tubes that contained EDTA, using a laser-based haematological analyser (Coulter Electronics, Hialeah, FL, USA). The white blood cell differentials were measured manually on Wright-stained blood smears.

We tested serum samples for sodium, potassium, chloride, calcium, phosphorus, partial pressure of oxygen, partial pressure of carbon dioxide, total carbon dioxide, and pH with an i-STAT Portable Clinical Analyzer (i-STAT Corportation, Princeton, NJ, USA). Concentrations of albumin, amylase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ glutamyltransferase, glucose, cholesterol, total protein, total bilirubin, urea nitrogen, and creatinine were measured with a Piccolo Point-Of-Care Blood Analyzer (Abaxis, Sunnyvale, CA, USA).

We undertook all coagulation assays in accord with the manufacturers' directions. Plasma concentrations of tissue-type plasminogen activator antigen, urokinase-type plasminogen activator, factor VIII, and complexes of tissue factor pathway inhibitor/factor Xa (TFPI/fXa) were ascertained by ELISA (American Diagnostica, Greenwich, CT, USA); we also measured plasma concentrations of D dimers by ELISA (Diagnostica Stago, Parsippany, NJ, USA). We ascertained plasma concentrations of protein C with a chromatic hydrolysis assay (DiaPharma, West Chester, OH, USA). Tissue factor activity in plasma was measured by fluorogenic cleavage assay (American Diagnostica).

We assayed cytokine and chemokine concentrations in monkey sera or plasma with commercially available ELISA kits, according to manufacturers' directions. Cytokines and chemokines assayed included monkey interleukin 10, interferon γ, and tumour necrosis factor (TNF) α (BioSource International, Camarillo, CA, USA). ELISAs for human proteins known to be compatible with rhesus macaques included interferon α and interferon β (BioSource), and interleukin 6 and monocyte chemoattractant protein-1 (MCP-1; R&D Systems, Minneapolis, MN, USA).



Number at risk					
rNARc2	9	9	7	3	3
Placebo	17	17	1	1	1

Figure 1: **Kaplan-Meier curves comparing survival of rhesus monkeys infected with Ebola virus and treated with placebo or rNAPc2**

*Placebo group supplemented with 14 historical controls.

We obtained tissues from the monkeys and immersion-fixed them in 10% neutral-buffered formalin and processed them for histopathology, immunohistochemistry, and electron microscopy, according to conventional methods.[2] Replicate sections of spleen, liver, and kidneys were stained with phosphotungstic acid-haematoxylin (PTAH) to show polymerised fibrin.[2]

Statistical analysis

We analysed our data by Fisher's exact test, and judged a p value of less than or equal to 0·05 significant.

For ethical reasons, use of relevant historical controls was required by the USAMRIID Laboratory Animal Care and Use Committee to reduce the number of non-human primates needed. We therefore used 14 rhesus monkeys inoculated with the same isolate and dose of Ebola virus, by the same route, and from contemporary studies to supplement the three Ebola virus-positive controls in this study to increase statistical power for comparing survival. Additionally, we supplemented these 17 untreated controls with 18 rhesus monkeys used on various therapeutic treatment studies that were inoculated with the same isolate and dose of virus, and by the same route, to compare survival rates. We used archived samples from three or four of the 14 untreated rhesus monkeys inoculated with the same isolate and dose of Ebola virus, and by the same route, to supplement the three virus-positive controls in this study for haematology, clinical chemistry, and coagulation assays.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We studied 12 healthy adult rhesus macaques, eight of which were female and four male. We treated three rhesus

monkeys with rNAPc2 and one with sterile saline 24 h after challenge with Ebola virus. The monkey treated with placebo died on the eighth day after challenge. By contrast, one monkey (female) treated with rNAPc2 survived, and the other two died on the 11th and 14th days after challenge. The animal that survived challenge has remained healthy for more than 1 year. We next administered rNAPc2 within 10 min of Ebola virus challenge to confirm the findings of our initial experiment and to attempt to achieve an additional beneficial effect. Two untreated controls died within nine days of receipt of Ebola virus. Four of six animals immediately treated with rNAPc2 died on days 8, 10, 13, and 14 post-infection; two animals (males) are still alive and healthy 9 months after challenge.

There was no apparent difference in survival or delay of death between animals treated with rNAPc2 24 h after exposure (one of three survived) or immediately after challenge (two of six survived). Mean survival for the six treated animals that died was 11·7 days compared with 8·3 days for untreated and historical controls; this prolongation in survival of the rNAPc2-treated monkeys was significant (p=0·0184; figure 1). Analysis of survival of the limited number of rNAPc2-treated and untreated monkeys suggested a beneficial effect (p=0·1039). However, comparison of the rNAPc2-treated animals with historical controls confirmed a significant increase in survival after treatment (p=0·0226).

Figure 2 shows the titres of Ebola virus in the plasma of infected monkeys. The three rNAPc2-treated animals that survived became viraemic, and subsequent testing by ELISA and neutralisation assay showed that these animals seroconverted to Ebola virus. By post-infection day 41, all three survivors had antibody titres against Ebola virus of one in 32 000 and $PRNT_{80}$ (80% plaque reduction neutralisation test) values of 1 in 40. Because the plasma viraemia concentrations noted on day 6 post infection in the rNAPc2-treated monkeys (mean 3·96 $\log_{10}$ PFU/mL) were

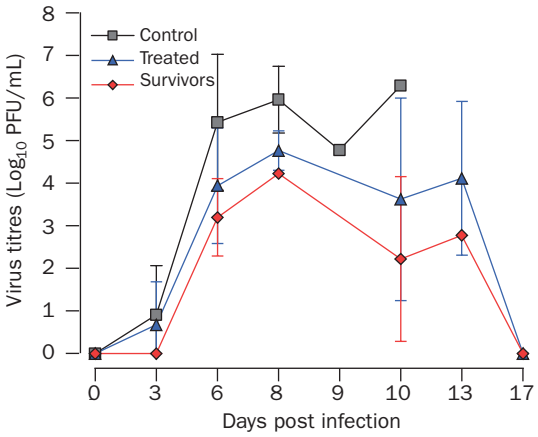


Figure 2: **Effect of treatment with rNAPc2 on Ebola virus plasma viraemia**
Data are mean (SD).

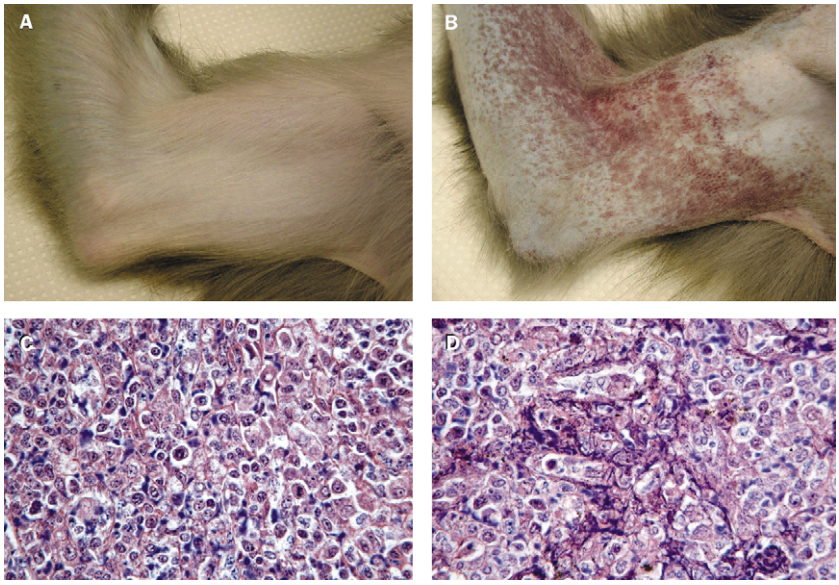


Figure 3: **Gross appearance and histological characteristics associated with rNAPc2-treatment of rhesus monkeys infected with Ebola virus**
A=typical right arm of rNAPc2-treated survivor at day 10 post infection. B=severe petechial rash of right arm of untreated positive control at day 9 post infection. C=PTAH stain of spleen from rNAPc2-treated monkey euthanised when moribund on day 13, showing no evidence of polymerised fibrin. D=PTAH stain of typical spleen from untreated positive control monkey euthanised when moribund on day 9, showing abundance of polymerised fibrin.

significantly lower than in untreated and historical controls (5·46 $\log_{10}$ PFU/mL; p=0·0319), we tested the antiviral activity and toxicity of rNAPc2 in vitro with established methods.[4] The presence of rNAPc2 did not prevent the development of virus-induced cytopathic effect at any of the concentrations used (range 0·045–100 μg/mL) nor did rNAPc2 show any evidence of toxicity (IC_{50} >100 μg/mL and TC_{50} >100 μg/mL).

With respect to clinical symptoms, cutaneous macular rashes indicative of coagulation abnormalities[2] were much less striking and were slower in developing in rNAPc2-treated animals than in untreated controls (figure 3). In fact, mild macular cutaneous rashes were only observed on the rNAPc2-treated animals just before death, but mild to severe rashes appeared on all untreated controls several days before death. None of the three treated monkeys that survived developed macular rashes. By comparison, all untreated and historical controls developed rashes.

Three of six rNAPc2-treated female macaques showed evidence of bleeding from the vagina shortly after challenge with Ebola virus; two of these animals showed evidence of anaemia with red blood cell, haematocrit, and haemoglobin values dropping from preinfection concentrations. We are uncertain as to the role of rNAPc2 in vaginal bleeding or anaemia, since we have noted vaginal bleeding and anaemia in untreated Ebola virus-infected female rhesus macaques in other studies. With the exception of one animal, there were no apparent differences in platelet counts between rNAPc2-treated monkeys and untreated controls; concentrations dropped equally in all infected macaques.

Changes in serum biochemistry in treated and untreated animals were limited to two enzymes frequently associated with impaired hepatic function. By post-infection day 6, increased concentrations of alkaline phosphatase were seen in all six untreated and historical controls and in all six rNAPc2-treated monkeys that succumbed to infection, but were not detected in the any of the three treated animals that survived challenge. We noted increased concentrations

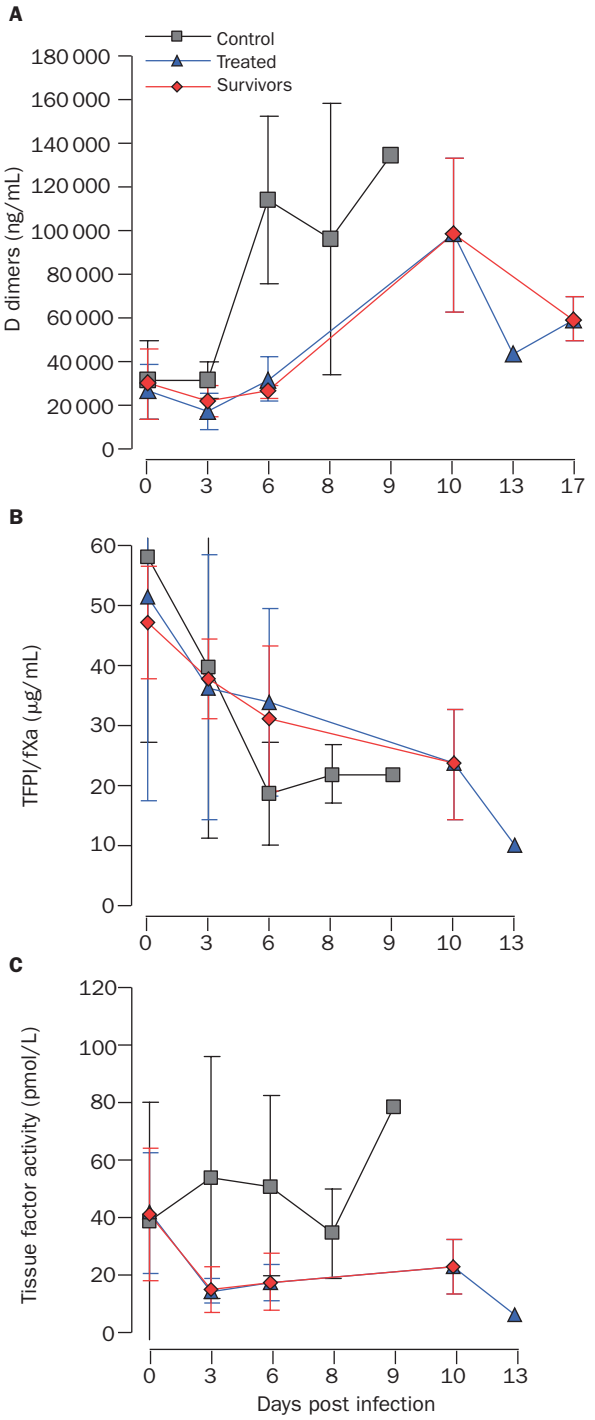


Figure 4: **Effect of treatment with rNAPc2 on coagulation responses during infection of rhesus monkeys**
Data are mean (SD).

of alanine aminotransferase in five of six untreated macaques and in five of six rNAPc2-treated monkeys; concentrations of alanine aminotransferase remained constant in the three animals that survived infection.

To ascertain the effect of rNAPc2 on the development of coagulopathy during Ebola haemorrhagic fever, we monitored a range of factors involved in regulation of coagulation and fibrinolysis. Assays done were chosen based on compatibility with macaques and obtaining the most useful data possible while conserving samples. For example, we did not measure fibrin degradation products because they do not discriminate between fibrinogen-derived and fibrin-derived split products, and are only an indicator that plasmin was present and that the fibrinolytic system was activated. As a more useful alternative, we measured D dimers, which are cross-linked fibrin degradation products. A positive D-dimer test indicates that both the clotting and fibrinolytic systems are activated, and raised concentrations of D dimers are noted in about 95% of all cases of disseminated intravascular coagulation.[14]

We noted significant changes in plasma D-dimer concentrations between the untreated controls and the rNAPc2-treated monkeys at days 3 ($p=0{\cdot}0032$) and 6 ($p<0{\cdot}0001$) after infection (figure 4). Increased plasma concentrations of D dimers were observed in all seven untreated and historical infected control monkeys and in two of nine rNAPc2-treated monkeys by day 6. There was a delayed effect in the development of D dimers in the rNAPc2-treated animals that survived infection, since two of three animals had increased D dimers only on day 10, and the other surviving animal never had raised concentrations of D dimers.

Although TFPI/fXa complex concentrations have been reported to vary considerably in disseminated intravascular coagulation, subnormal TFPI-fXa complex values have been attributed to acute coagulopathy, and are presumed to be the result of consumption of TFPI.[15] This consumption of TFPI has been further associated with a reduced capacity to inactivate the fVIIa/tissue factor complex.[15] Consistent with these observations, TFPI-fXa complex concentrations were significantly lower in untreated monkeys versus rNAPc2-treated animals at day 6 post infection ($p=0{\cdot}0360$; figure 4).

We noted significantly lower plasma tissue factor activity concentrations in the rNAPc2-treated monkeys than in the untreated controls on days 3 ($p=0{\cdot}0141$) and 6 ($p=0{\cdot}0068$) post infection (figure 4), with tissue factor concentrations declining in seven of nine treated animals tested on day 6 and showing no change or slightly increasing in six of seven untreated and historical controls. These results indicate that rNAPc2 is biologically active in Ebola virus-infected monkeys, but do not necessarily show a reduction in the overall perturbation of the coagulation cascade. Plasma concentrations of tissue-type plasminogen activator antigen and urokinase-type plasminogen activator were similar to results shown for D dimers, with concentrations of both seemingly higher in the untreated and historical controls at day 6 post infection than in the rNAPc2-treated monkeys. Plasma protein C values did not drop below 77% of baseline values in any of the three surviving monkeys, though drops below 60% were noted in treated and untreated animals that succumbed to infection at terminal time points. Plasma concentrations of factor VIII transiently declined at day 6 post infection in six of six rNAPc2-treated monkeys tested and in all five untreated and historical controls. Concentrations of factor VIII increased to or above day-6 values at terminal time points (days 8 or 9) in all four untreated and historical controls, and between days 8 and 13 in all three rNAPc2-treated animals tested. Increases and decreases in factor VIII concentrations have been reported in disseminated intravascular coagulation. Notably, transient decreases in factor VIII concentrations have been described in the acute stage of dengue haemorrhagic fever.[16]

To ascertain the extent to which rNAPc2 blocked the formation of fibrin, we did a histological assessment of the six treated animals that succumbed to infection. Two of these animals lacked the prominent fibrin deposits and intravascular thromboemboli noted in the untreated and historical controls (figure 3). The other four treated animals showed varying degrees of fibrin deposits and intravascular thromboemboli.

Activation of coagulation and inflammation during severe infection seems to be linked in a bimodal way. While cytokines are involved in the procoagulant state that follows endotoxaemia or severe infection, results of many studies[17,18] have shown that activated coagulation factors in turn are capable of eliciting a proinflammatory response. Activation of coagulation in healthy people by the administration of recombinant factor VIIa also elicits a large increase in the concentrations of several cytokines, including interleukin 6.[19] This increase is absent when patients are pretreated with rNAPc2.[19] Moreover, rNAPc2 attenuates the interleukin 10 response in experimental human endotoxaemia.[20] Therefore, we investigated the cytokine and chemokine responses of rNAPc2-treated monkeys versus untreated controls, since many of these inflammatory mediators can play an important part in the interaction between coagulation and systemic inflammation during infections. On day 6 post infection, we detected increased concentrations of interleukin 6 in three of nine rNAPc2-treated monkeys and in all seven untreated infected controls (figure 5). Increased concentrations of interleukin 6 were not detected in the three monkeys that survived infection. Similarly, we noted increased amounts of MCP-1 in four of nine rNAPc2-treated monkeys and in all seven infected untreated monkeys at day 6 (figure 5). No increase in plasma concentrations of MCP-1 was detected in the three surviving monkeys. Plasma or sera concentrations of interleukin 10 and other cytokines (interferon α, interferon β, interferon γ, TNFα) did not differ greatly between groups.

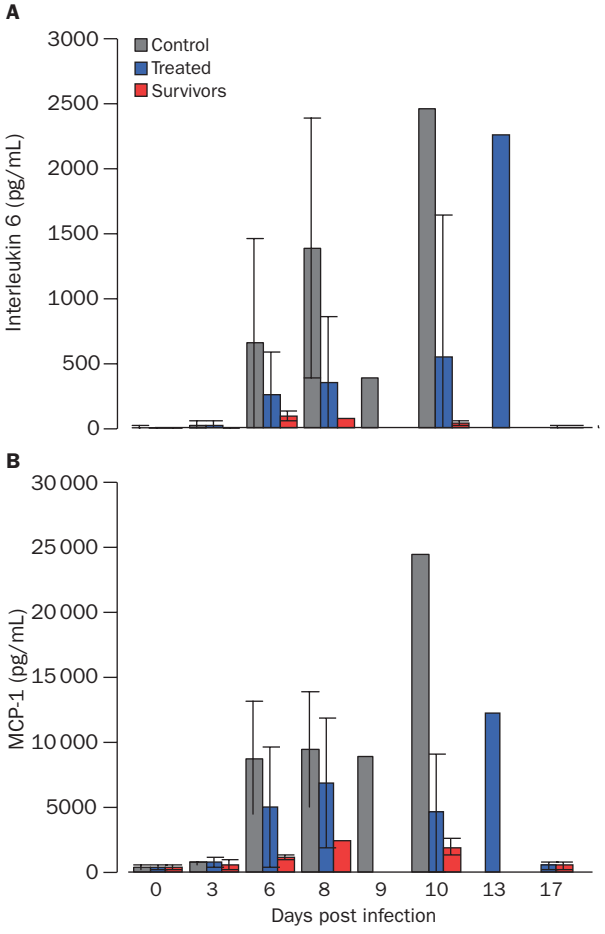


Figure 5: **Effect of treatment with rNAPc2 on inflammatory markers during infection of rhesus monkeys**
Data are mean (SD).

Discussion

Our findings show that treatment with rNAPc2 results in significant survival of non-human primates infected with the Ebola virus. Survival of rNAPc2-treated macaques was associated with reduced activation of coagulation and fibrinolysis, and with attenuation of the systemic proinflammatory response as evidenced by low concentrations of interleukin 6 and MCP-1. Since two of the treated animals showed evidence of anaemia, possibly triggered by bleeding from the uterine lining, we postulate that a more aggressive clinical-care strategy—eg, transfusion—would further increase survival rates when used in conjunction with rNAPc2.

Our results have great clinical implications, since our treatment approach of Ebola haemorrhagic fever targets the disease process rather than replication of the infectious agent. Moreover, our findings raise the possibility that rNAPc2 could be useful in the treatment of other viral haemorrhagic fevers. Importantly, rNAPc2 has a suitable phamacokinetic and safety profile in human beings.[12] The clinical efficacy of this treatment modality now needs to be proven.

Contributors

T W Geisbert designed, planned, and coordinated the study, treated animals, gathered study samples, did coagulation and immunological assays, and wrote the manuscript. L E Hensley participated in the study design, undertaking of coagulation and immunological assays, interpretation of results, and writing of the manuscript. J B Geisbert played the substantial part in undertaking the infection experiments, treating animals, and gathering study samples, and also did virological and immunological assays. T Larsen did the pathological assessment of the samples. J Paragas did antiviral activity analyses. P B Jahrling, H A Young, T E Fredeking, W E Rote, and G P Vlasuk were involved in the design and undertaking of the study, interpretation of data, and writing of the manuscript.

Conflict of interest statement

G P Vlasuk and W E Rote have filed for a US patent for rNAPc2.

Acknowledgments

We thank Denise Braun, Jeff Brubaker, and Jeff Smith for their expert technical assistance. We also thank Kelly Davis, Michael Hensley, James Swearengen, and Catherine Wilhelmsen for helpful discussions and comments, and Paul Gibbs and Sarah Norris for skilful assistance with statistical analysis of data. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US army.

This study was funded by the Medical Chemical/Biological Defense Research Program, US Army Medical Research and Material Command (project number 02–4-4J-081).

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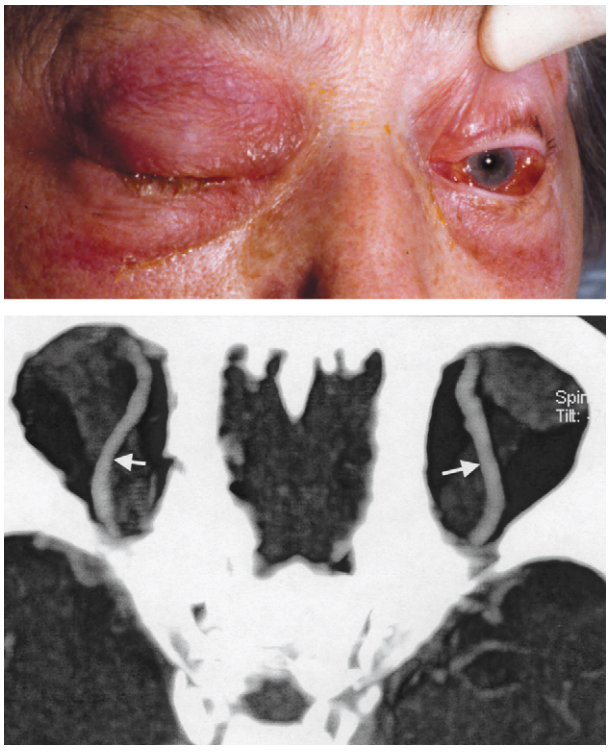
Clinical picture

Cavernous sinus thrombosis

Josef G Heckmann, Bernd Tomandl



An 82-year-old woman had transphenoidal resection of an inactive pituitary adenoma, and was given low-dose heparin. She developed secondary hypothyroidism, and was treated with L-thyroxine. 1 year later she was given low-dose heparin again, after conservative care of diverticulitis. 9 days later, she developed reduced ocular motility, bilateral exophthalmus, and conjunctival chemosis (figure, upper). CT angiography confirmed the diagnosis of cavernous sinus thrombosis by showing enlarged bilateral superior ophthalmic veins (figure, lower, arrows). Her platelet count was 20×10^9/L. A heparin-induced platelet activation assay was positive. Heparin-induced and danaparoid-induced IgG antibodies were present. We replaced heparin with intravenous recombinant hirudin, and she improved over the next 3 days.

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